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Uptake by Roots and Translocation to Shoots of Two Morpholine Fungicides in Barley

Keith Chamberlain, Shilpa Patel & Richard H. Bromilow

IACR-Rothamsted, Harpenden, Herts AL5 2JQ, UK

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Abstract: Despite being lipophilic, morpholine fungicides are systemic in plants. Such transport may be explicable by their protonation (pKa ~ 7·5) at the pH of plant compartments to yield the more polar cation. This behaviour might be a useful attribute to be incorporated into other classes of lipophilic pesticides. To understand quantitatively the behaviour of the morpholine fungicides, the uptake by roots and transport to shoots in barley of two such ^{14}C-labelled compounds, dodemorph and tridemorph, were investigated using bathing solutions of differing pH. At pH 5, uptake and transport were small, but increased by approximately two orders of magnitude at pH 8. Tridemorph, the more lipophilic of the two compounds, was highly accumulated by roots at pH 8 and moderately translocated to shoots. In contrast, dodemorph was translocated to shoots at pH 8 with remarkable efficiency, moving into the xylem across the endodermis at 23 times the efficiency of water, though accumulation in roots was less than that of tridemorph. Behaviour at 24 h was largely similar to that at 48 h for both compounds, indicating that uptake and translocation are equilibrium processes maintained over time. Transport to shoots for each compound was directly proportional to the concentrations accumulated in the roots, except at low pH where partitioning into root solids became proportionately more important with such material not being directly available for transport to the xylem across the endodermis. Uptake and transport of these basic fungicides are explained in terms of their partitioning and of their accumulation in acidic plant compartments by ion trapping as the protonated form; this behaviour is discussed in relation to the pKa and lipophilicity of these compounds. © 1998 Society of Chemical Industry

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1 INTRODUCTION

The first classes of agricultural fungicides marketed had only a contact and protectant action when applied to plants. However, by the 1960s new classes of fungicides had been discovered that had effective systemic and curative properties. The activity of morpholines with large alkyl groups attached was first reported by König *et al.*[1] in 1965, and by the end of that decade BASF had introduced two such compounds, dodemorph and tridemorph.[2–4] These are systemic fungicides that move in the xylem with the transpiration stream and act by inhibiting ergosterol biosynthesis.

The movement of these compounds in plants is interesting, as their physicochemical properties are unusual in pesticides. Morpholines are moderately strong bases, having pKa $\sim$ 7·5, such that both free base and its protonated form will be present in appreciable proportions in plant compartments, whose pH ranges from $\sim$ 5 in the apoplast to $\sim$ 8 in the phloem sap. The free base forms of these two fungicides are quite lipophilic, with log $K_{ow} > 5$ (K_{ow} is the 1-octanol/water partition coefficient). For non-ionised compounds, only pesticides with log $K_{ow} < 4$ are systemic in plants, with optimum uptake by roots and translocation to shoots occurring for compounds of log K_{ow} 1 to 3.[5–9] The systemicity of the morpholine fungicides thus appears to involve their cations crossing plant membranes, as such charged species are substantially more polar than the free base; certainly the cation will be the major species present (>99%) and translocated in xylem sap of pH 5.

However, Hsu *et al.*[10] showed that the permeation rates through plant membranes of a series of quaternised pyridines (permanent organic cations) were rather small, indeed so small that these compounds were sufficiently retained in the slow-moving phloem sap to give measurable symplastic movement. These cations would be more polar than the cations from the morpholine fungicides, and so it was not certain whether the fungicide cations would also permeate membranes slowly. Furthermore, the fungicide dodine[11] is also systemic to some extent, this compound having both a long alkyl chain and a highly basic guanidine group (pKa $\sim$ 12) such that it will be present predominantly as the cation in all plant compartments.

The factors controlling the systemicity of these compounds are thus unusual, and understanding them might allow the introduction of systemicity into other classes of lipophilic contact pesticides such as some insecticides. This work reports on the uptake by barley roots and subsequent transport to shoots of dodemorph and tridemorph, using solutions buffered over a range of pH. A companion paper[12] reports similar studies but utilising two series of bases spanning a range of pKa and log K_{ow} such that the role of these physicochemical properties in determining the systemicity of bases in plants can be elucidated.

2 MATERIALS AND METHODS

2.1 ^{14}C-Labelled dodemorph and tridemorph

The fungicides were supplied by BASF Aktiengesellschaft. Dodemorph (4-cyclododecyl-2,6-dimethylmorpholine) and tridemorph (main component 2,6-dimethyl-4-tridecylmorpholine) (Fig. 1) were technical materials. [*morpholin*-2,6-^{14}C]Tridemorph and [*morpholin*-2,6-^{14}C]dodemorph had radiochemical purities of 99·7 and 98·0% respectively, and were used at specific activities of approximately 34 MBq mmol^{-1}.

2.2 pKa and 1-octanol/water partition coefficients (K_{ow})

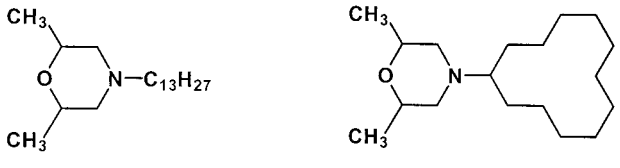
These are given in Fig. 1 and are literature values.[13] Tridemorph is the slightly more lipophilic, whilst being a weaker base than dodemorph.

2.3 Growth and treatment of barley plants

Seeds of barley (*Hordeum vulgare* L. cv. Alexis), germinated on moist tissue paper, were transferred to aerated nutrient solution (half-strength Hoaglands[14]) and grown in a controlled environment with a 16-h day (10 klux) at 20°C and 8-h night at 16°C.[5]

To measure the uptake over 24 and 48 h of the compound under test, groups of 10-day-old plants were transferred to buffer solution (100 ml) containing the compound at an initial concentration of 5 μM. Transpiration was measured by weighing the vessel and plants at the beginning and end of each test, when the concentration of the chemical in solution was also measured.

Uptake was measured at pH values from 5·0 to 8·0, with two replicates (each of six plants) at each of the four pH values used. Solutions were buffered with 0·005 M citric acid/sodium citrate (pH 5·0), 0·005 M sodium dihydrogen phosphate/monohydrogen phosphate (pH 6·0 and 7·0) and 0·01 M TRIS (2-hydroxy-1,1-bis(hydroxymethyl)ethylamine) (pH 8·0). The pH values were checked during the tests at 12, 24 and 36 h, and adjusted back to the original pH if necessary



tridemorph
log K_{ow} 5.6, pKa 7.4

dodemorph
log K_{ow} 5.2, pKa 7.8

Fig. 1. Structures and physicochemical properties of dodemorph and tridemorph.

by addition of a little 1·0 M sodium hydroxide or hydrochloric acid; this procedure gave pH control within ± 0·2 pH unit. At pH 8·0, uptake by roots of both fungicides was sufficiently large that the solutions rapidly became substantially (about 50%) depleted; these sets of plants were transferred after 2 h to fresh solutions at the initial 5 μM concentration.

2.4 Partition onto macerated barley roots

Freeze-dried barley roots (0·010 g, equivalent to 0·179 g fresh weight) were gently shaken with a 5 μM aqueous solution (2·5 ml in duplicate) of the ^{14}C-labelled fungicide for 2 h buffered as described above to pH 5, 6, 7 and 8. The mixture was then centrifuged and the concentration of fungicide measured in the supernatant solution (0·5 ml in duplicate) by liquid scintillation counting (LSC). Some sorption to glass occurred, especially for tridemorph at the higher pH values, and corrections were made for this.

2.5 Extraction and measurement of ^{14}C-labelled fungicides in barley roots and shoots

The combined root or shoot samples from the six barley plants were macerated with portions of acetone, and the extracts filtered through cotton wool into a volumetric flask (100 ml). After making up to volume, duplicate aliquots (5·0 ml) were evaporated to near dryness in a round-bottomed flask on a rotary evaporator with a bath temperature not exceeding 30°C; problems were encountered with losses of tridemorph by volatilisation, and so digol (0·05 ml) was added as a keeper to the aliquot before evaporation of these samples.

The residue was transferred in a little acetone to the non-sorbent application strip of a 19-band silica gel 60A TLC plate, size 20 × 20 cm which was then developed in hexane + acetone + ethanol (80 + 10 + 10, by volume). Cold standards were visualised using an iodine tank, and the tracks of ^{14}C-labelled fungicides were scanned for 15 min using a Berthold Tracemaster 20 linear analyser. Dodemorph had R_F 0·80 and tridemorph, despite being a mixture of analogues, ran as a single spot (R_F 0·62) in this solvent, whereas in a second solvent tested (hexane + acetone, 91 + 9 by volume) it separated into three spots, which was less convenient for quantification purposes.

The band of parent material was scraped off and radioactivity determined by liquid scintillation counting for 10 min using Ultima Gold cocktail (Packard; 9 ml) and a Kontron Betamatic Counter with quench correction using an external standard. Recoveries were generally better than 85% though were variable with tridemorph since use of the digol keeper tended to impair the TLC separation. To confirm the tridemorph results, a second set of extractions was done using methanol (50 ml) containing hydrochloric acid (0·5 M; 1·0 ml) and an aliquot (1·0 ml concentrated to 100 μl) taken for radioactivity measurement. A second aliquot was subjected to TLC and scanning as above, and the proportion of radioactivity associated with the parent compound was used to correct the total radioactivity; this procedure gave a consistent recovery of 91%. All results are corrected for efficiency of recovery.

2.6 Calculation of results

Uptake by roots was presented as an accumulation factor defined as the Root Concentration Factor (RCF).[5,15,16]

$$\mathrm{RCF} = \frac{\text{concentration in roots (}\mu\text{g g}^{-1}\text{ fresh wt)}}{\text{concentration in external solution (}\mu\text{g ml}^{-1}\text{)}}$$

where the concentration in solution was that at sampling (24 or 48 h). For the partitioning onto the freeze-dried, macerated roots, the $\mathrm{RCF}_{\mathrm{macerated}}$ was also calculated on a fresh weight basis. Efficiency of transport to shoots was calculated as the Transpiration Stream Concentration Factor (TSCF):[5,15,16]

$$\mathrm{TSCF} = \frac{\text{concentration in xylem (}\mu\text{g ml}^{-1}\text{)}}{\text{concentration in external solution (}\mu\text{g ml}^{-1}\text{)}}$$

$$= \frac{\text{amount accumulated in shoots (}\mu\text{g)}}{\text{vol. transpired (ml)} \times \text{solution conc. (}\mu\text{g ml}^{-1}\text{)}}$$

where the concentration of the external solution was taken to be the mean of the initial and final concentrations. The TSCF values have not been corrected for any degradation in the shoot (see Section 3.2). All RCF and TSCF results are presented as the mean of the two replicates, each of six plants combined.

3 RESULTS AND DISCUSSION

3.1 Uptake by barley roots from solution

Both dodemorph and tridemorph were well taken up by barley roots and translocated to shoots, these processes being very sensitive to solution pH over the range pH 5 to 8 and being most efficient at higher pH (Fig. 2). The mass balances were generally over 85% except for tridemorph at pH 7 and 8 where losses by sorption to the glass vessels reduced the balances to around 72%.

Uptake by roots, expressed as the accumulation factor RCF, was very similar (except at pH 5) over the uptake periods of 24 and 48 h (Fig. 3; note all graphs are plotted semi-logarithmically), indicating that it is an

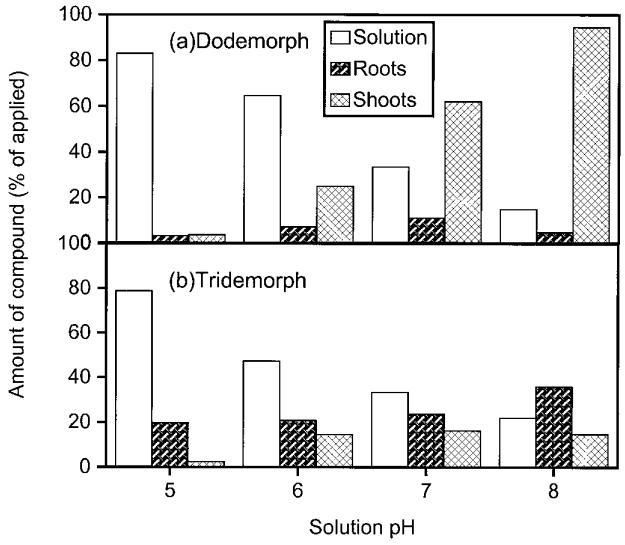


Fig. 2. Uptake from solution over 24 h and distribution of morpholine fungicides in barley; (a) dodemorph (b) tridemorph.

equilibrium that is rapidly reached and then maintained. Similar observations were reported for the RCF of non-ionised compounds[5] and of weak acids[17,18] in barley and other plants. Tridemorph was the more strongly taken up by roots, reaching an RCF of 183 at pH 8 compared to only 49 for dodemorph at this pH. However, at pH 5.0, the RCF increased between 24 and 48 h, markedly so for tridemorph; this is attributed to the roots becoming acidified at this low pH.

Weak acids can likewise be accumulated in plant roots,[17,18] with such uptake and subsequent translocation here being greatest from solutions of low pH. This behaviour has been explained in terms of ion trapping,[17,19] whereby the differential permeation rates of the non-dissociated form and its respective anion allow acids to be accumulated in plant compartments of high pH, *viz* the symplast, including the phloem. At solution pH values of <5, acidification of the root cells with time reduced uptake of acids by ion trapping,[17] in

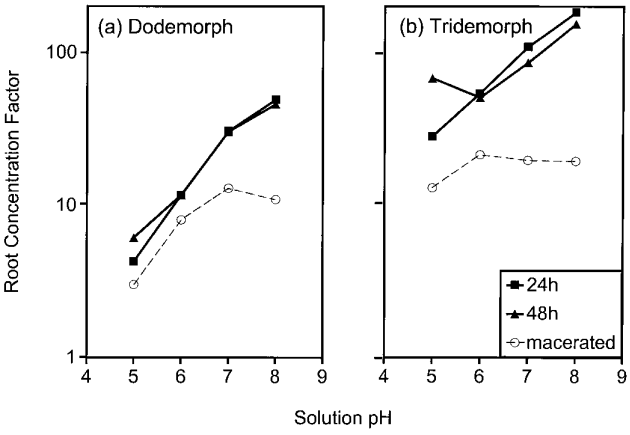


Fig. 3. Uptake by barley roots from solutions of various pH values over 24 and 48 h; (a) dodemorph (b) tridemorph. Dashed lines indicate partitioning onto macerated barley roots over 2 h.

agreement with the increased uptake of the morpholines noted above. The compounds are not specifically recognised by any transport carriers but the plant expends energy by proton-pumping to maintain its internal pH differences which the passive diffusion of the acids tends to dissipate.

Such a process can equally well explain the accumulation of these fungicidal bases in plant compartments, save that the presumed higher permeability of plant membranes to the non-ionised form compared to its respective cation now permits accumulation in the acidic compartments, *viz* the vacuole and xylem. When the external solution is of low pH, around 5 to 6, which is close to the apoplast pH, then such ion trapping is very weak, but if the solution pH is raised to 8 then ion trapping becomes a dominating process.

However, two other processes may contribute to uptake of these bases by plant roots. The first of these is the Nernst effect, whereby the negative charge of around $-0{\cdot}12$ V on the plasmalemma membrane which surrounds the cell leads to the strong accumulation of cations; however, for ionisable bases, leakage of the non-ionised species can undermine such accumulation. The free-base forms of these morpholine fungicides are thought to permeate membranes well as indicated by the high TSCF values (see Section 3.2) observed in these tests, and so such charge effects would be very small. It should be noted that the plasmalemma potential is sensitive to the K^+ status of the plant;[20,21] although K^+ concentrations were not maintained in our experiments after transfer to the fungicide solutions, the similar RCF values at 24 and 48 h indicate that this was not a complicating factor.

The second possible process is partitioning onto the root solids of plants, which gives an appreciable contribution to RCF for non-ionised compounds of $\log K_{ow} > 2$ and also for the strongly basic phenethylamines (pKa $\sim 9{\cdot}5$) of $\log K_{ow} > 2$.[12] The possible contribution of partitioning was investigated using macerated freeze-dried roots; modest partitioning was observed, which remained constant for each compound at pH 7 and 8 but decreased at lower pH (Fig. 3). Tridemorph was more strongly partitioned than dodemorph, as might be expected from its higher lipophilicity ($\log K_{ow}$ 5·6 compared to 5·2); however, partitioning even at pH 8 was an order of magnitude less than would be expected for a non-ionised compound of comparable lipophilicity. This partitioning was similar to the RCF at pH 5 for the intact plants, but less at higher pH due to ion trapping becoming here the dominant uptake process.

3.2 Translocation to shoots

Translocation to shoots was very efficient, again increasing markedly with solution pH (Fig. 4) as

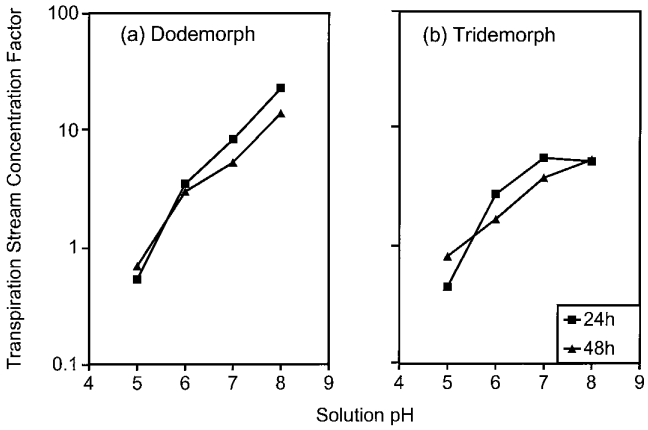


Fig. 4. Translocation to barley shoots following uptake by roots from solutions of various pH values over 24 and 48 h; (a) dodemorph (b) tridemorph.

observed for the RCF. Dodemorph was remarkably well translocated at pH 8·0, with a TSCF of 23 at 24 h. This means that dodemorph was entering the xylem at 23 times the rate of water, which behaviour may be compared to the maximum TSCF value of 1·0 thought to be achievable by non-ionised compounds and attributable to passive diffusion alone.[5] Even the weak acids exhibiting ion trapping did not have TSCF values that exceeded 3·0 within the tested pH range of 4 to 8.[17]

Except at pH 5·0, the TSCF values at 48 h were somewhat lower than at 24 h, even though the RCF was constant over these time periods at pH 6·0 to 8·0. Two factors probably contribute to this difference. Firstly, the strong uptake of these compounds meant that the external solution concentration declined markedly over 48 h, and the correction applied for this and which assumes a linear decline in concentration[5] may be inadequate. Secondly, and perhaps the main reason, is that we have measured TSCF indirectly by assessing accumulation in shoots over time and for a known transpiration; any metabolism of compound in the shoots would thus lead to the true TSCF being underestimated,[5] and such an error would be increased with the longer uptake period. Nonetheless, the reasonable mass balances over both 24 and 48 h and the only small amounts of extractable metabolites seen by TLC indicate that such metabolism was not a major problem, and that the TSCF values measured over 24 h are close to the true values. At pH 5·0, TSCF values were higher at 48 than 24 h for both compounds, paralleling the increase in RCF observed with time and attributed to acidification of the roots by 48 h.

The TSCF was proportional to the RCF, this factor being essentially constant for each compound whatever the solution pH (Fig. 5). From this we infer that the movement into the xylem is proportional to the overall concentrations in the roots. The TSCF/RCF ratio only fell slightly at the lowest solution pH studied, and the probable explanation for this is that at pH 5·0 uptake into the aqueous compartments of roots by ion trapping is small; thus the contribution of the RCF partitioning onto root solids is proportionately greater than at higher solution pH values and, as observed with non-ionised compounds, such partitioned material is not directly available for movement into the xylem. This ratio is an order of magnitude greater for dodemorph than tridemorph, and indeed for dodemorph was about twice that observed for amines of similar pKa but log $K_{ow} \sim 2$.[12]

4 CONCUSIONS

Dodemorph and tridemorph, two lipophilic morpholine fungicides of base pKa 7·8 and 7·4 respectively, were well taken up from solution by barley roots and translocated to shoots. Uptake by roots (as RCF) was generally similar at 24 and 48 h of exposure, indicating an equilibrium process, whereas rates of accumulation in shoots (as TSCF) appeared to slow slightly from 24 to 48 h; this was probably due to some metabolism in the shoots. However, at pH 5·0, acidification of the roots with time led to rises in RCF and TSCF values between

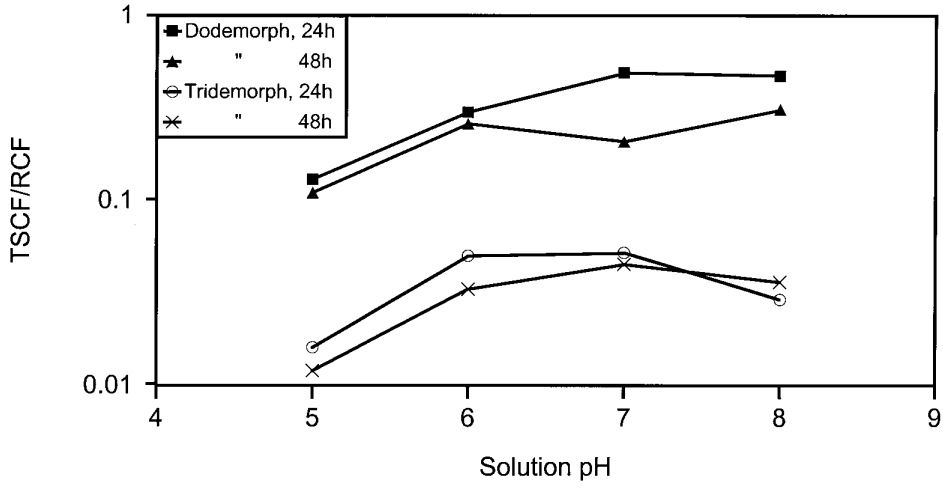


Fig. 5. Ratio of TSCF/RCF after 24 and 48 h of uptake for dodemorph and tridemorph.

24 and 48 h. Uptake and transport for each compound both increased by approximately an order of magnitude as pH was increased from pH 5 to 8. At pH 8, dodemorph had a remarkably high TSCF of 23, indicating that its movement into xylem was 23 times faster than the corresponding entry of water across the endodermis. Tridemorph was about one-quarter as efficiently translocated as dodemorph at this pH, though its accumulation by roots was more than three times higher, with an RCF of 183, after 24 h. Some of the uptake into roots can be attributed to partitioning of these lipophilic bases onto root solids, but the major process, especially at the higher solution pH values, was ion trapping whereby the lower permeability of membranes to protonated bases compared to the free base permits accumulation in plant compartments of low pH, *viz* the apoplast, including the xylem. The TSCF/RCF ratio, indicative of the efficiency of movement from root-cell cytoplasm across the endodermis and into the xylem, was largely independent of solution pH for each compound, but was substantially higher for dodemorph than tridemorph. This better transport of dodemorph can be explained in terms of its physicochemical properties, having the lower K_{ow} which favours permeation together with the slightly higher pKa than tridemorph, both properties that favour ion trapping into the xylem.

A notable feature of these transport studies is the effectiveness of the ion trapping for such lipophilic molecules, indicating the ease with which they permeate membranes. In contrast, non-ionised pesticides of log $K_{ow} > 5$ are not translocated from roots to shoots, such lack of transport not being due to any 'filtering out' by partitioning, for example to plant roots, as the RCF does not increase with time; these compounds are thus continuously excluded from the xylem, this being done presumably by the surrounding ring of endodermal cells. The mechanism of this exclusion process is not clear, as permeation rates of compounds often increase with lipophilicity in non-plant membranes. It may be that the amphoteric nature of bases allows them to permeate both polar and non-polar regions of layered membranes, and that the surprisingly low partitioning into roots of these morpholines facilitates membrane permeation. These aspects of the transport behaviour of bases are investigated further with a wider range of compounds in a companion paper.[12]

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